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HIGHLIGHTED TOPIC | *Muscle Dysfunction in COPD*

Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD

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[1]*Department of Respiratory Medicine, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Center+, Maastricht, The Netherlands;* [2]*Pulmonology Department-Muscle and Respiratory System Research Unit (URMAR), IMIM-Hospital del Mar, Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona, Barcelona, Spain, and Centro de Investigación en Red de Enfermedades Respiratorias, Instituto de Salud Carlos III, Bunyola, Majorca, Balearic Islands, Spain*

van den Borst B, Slot IG, Hellwig VA, Vosse BA, Kelders MC, Barreiro E, Schols AM, Gosker HR. Loss of quadriceps muscle oxidative phenotype and decreased endurance in patients with mild-to-moderate COPD. *J Appl Physiol* 114: 1319–1328, 2013. First published July 19, 2012; doi:10.1152/japplphysiol.00508.2012.—Being well-established in advanced chronic obstructive pulmonary disease (COPD), skeletal muscle dysfunction and its underlying pathology have been scarcely investigated in patients with mild-to-moderate airflow obstruction. We hypothesized that a loss of oxidative phenotype (oxphen) associated with decreased endurance is present in the skeletal muscle of patients with mild-to-moderate COPD. In quadriceps muscle biopsies from 29 patients with COPD (forced expiratory volume in 1 s [FEV$_1$] 58 ± 16%pred, body mass index [BMI] 26 ± 4 kg/m^2) and 15 controls (BMI 25 ± 3 kg/m^2) we assessed fiber type distribution, fiber cross-sectional areas (CSA), oxidative and glycolytic gene expression, OXPHOS protein levels, metabolic enzyme activity, and levels of oxidative stress markers. Quadriceps function was assessed by isokinetic dynamometry, body composition by dual-energy X-ray absorptiometry, exercise capacity by an incremental load test, and physical activity level by accelerometry. Compared with controls, patients had comparable fat-free mass index, quadriceps strength, and fiber CSA, but quadriceps endurance was decreased by 29% (P = 0.002). Patients with COPD had a clear loss of muscle oxphen: a fiber type I-to-II shift, decreased levels of OXPHOS complexes IV and V subunits (47% and 31%, respectively; P < 0.05), a decreased ratio of 3-hydroxyacyl-CoA dehydrogenase/phosphofructokinase (PFK) enzyme activities (38%, P < 0.05), and decreased peroxisome proliferator-activated receptor-γ coactivator-1α (40%; P < 0.001) vs. increased PFK (67%; P < 0.001) gene expression levels. Within the patient group, markers of oxphen were significantly positively correlated with quadriceps endurance and inversely with the increase in plasma lactate relative to work rate during the incremental test. Levels of protein carbonylation, tyrosine nitration, and malondialdehyde protein adducts were comparable between patients and controls. However, among patients, oxidative stress levels were significantly inversely correlated with markers of oxphen and quadriceps endurance. Reduced muscle endurance associated with underlying loss of muscle oxphen is already present in patients with mild-to-moderate COPD without muscle wasting.

oxidative capacity; quadriceps endurance; muscle wasting; physical activity

SKELETAL MUSCLE DYSFUNCTION is a hallmark of advanced chronic obstructive pulmonary disease (COPD) and significantly contributes to decreased exercise capacity and poor quality of life (1, 5). Peripheral skeletal muscle wasting and a loss of oxidative phenotype (oxphen) are the two major myopathological findings recognized in patients with advanced COPD, being associated with decreased muscle strength (9) and endurance (2), respectively. Important to note is that studies investigating skeletal muscle dysfunction and its underlying pathology in COPD have been performed almost exclusively in patients with GOLD stages 3–4 with significant muscle wasting. In the INTERCOM trial, Van Wetering et al. (43) recently showed that patients with mild-to-moderate COPD (mean forced expiratory volume in 1 s [FEV$_1$] 60% of predicted) responded well to a lifestyle intervention program in terms of improvement in cycle endurance capacity and health status. Muscle strength and muscle mass of that study group were within the normal range, but no information was available regarding peripheral muscle endurance and markers of skeletal muscle oxphen.

Loss of peripheral muscle oxphen in advanced COPD includes a I-to-II fiber type shift and reduced activities of enzymes involved in oxidative energy metabolism (2, 15, 37). In addition, Remels et al. (33) previously showed that key oxphen regulators [peroxisome proliferator-activated receptors (PPARs), PPAR-γ coactivator-1α (PGC-1α), and mitochondrial transcription factor A (Tfam)] were reduced in muscles of patients with advanced COPD. Moreover, loss of muscle oxphen may render the muscle more susceptible to oxidative stress (21, 22, 31). Oxidative stress is believed to be an important player in skeletal muscle wasting and dysfunction in COPD (6, 10). However, data on muscle oxphen and oxidative stress in patients with COPD and milder degrees of airflow obstruction are scarce.

In the current study we compared markers of oxphen measured in biopsies of quadriceps muscle from patients with mild-to-moderate COPD and from healthy controls matched for age, sex, and body mass index (BMI). In addition, we explored associations between muscle metabolic profile and quadriceps function, exercise capacity, physical activity level, and skeletal muscle oxidative stress. We hypothesized that a loss of oxphen, associated with

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decreased endurance, is already present in skeletal muscle of patients with mild-to-moderate COPD.

METHODS

Subjects and study design. The study population comprised 29 patients with clinically stable mild-to-moderate COPD and 15 healthy controls. Patients were recruited from the outpatient clinic of the Maastricht University Medical Center+ (MUMC+, Maastricht, The Netherlands) and via advertisements in local newspapers. Patients were excluded if they were on long-term oxygen therapy, used oral prednisolone, or had an acute exacerbation of symptoms with hospital admission in the previous 8 wk and rehabilitation in the previous 6 mo. Patients with a known comorbidity that could potentially interfere with study outcome parameters were carefully excluded. These included diabetes, recent cardiovascular event, inflammatory bowel disease, obstructive sleep apnea, thyroid disease, and cancer. Healthy controls were recruited via advertising in local newspapers. The absence of these diseases in the healthy subjects was verified through history-taking by a physician and pulmonary function tests to verify the absence of airflow limitation. Care was taken to select a group of healthy controls with similar age, BMI, and sex distribution as the patients with COPD regardless of their daily physical activity level. Written, informed consent was obtained from all subjects and the ethical review board of the MUMC+ approved the study (08-2-059). The trial was registered at http://www.trialregister.nl as NTR1402.

On *day 1*, body composition and quadriceps function were assessed as described below, and subjects started wearing an accelerometer for 6 days to objectively quantify physical activity level. On *day 7*, the accelerometry data were retrieved, quadriceps tissue samples were obtained, pulmonary function was assessed, and an incremental load test was performed.

Pulmonary function, smoking status, and exercise capacity. Pulmonary function testing included forced spirometry and single-breath diffusion capacity measurement (Masterlab, Jaeger, Würzburg, Germany). Instruments were calibrated twice a day. All values obtained were expressed as a percentage of reference values (32). Smoking status was based on self-report, and those who had smoked or were current smokers were designated as ever-smokers. All subjects performed an incremental load cycling test to determine peak oxygen uptake ($\dot{V}O_2$ peak) and peak load (W peak) as previously described (12). Arterial punctures of the radial artery at rest and at $\dot{V}O_2$ peak were available from 19 patients with COPD and 11 healthy subjects. Arterial blood gas analyses and lactate concentrations were determined (Blood gas analyzer 865; Chiron Diagnostics, Emeryville, CA).

Anthropometry and body composition. Height in meters and weight in kilograms were assessed on a standard scale. BMI was calculated as weight/height^2. Whole-body dual-energy X-ray absorptiometry was performed to assess body composition as described (42). Fat-free mass index (FFMI) was calculated as fat-free mass/height^2. The prevalence of muscle depletion was explored by applying the criteria described by Schols et al. (38) (FFMI <16 kg/m^2 for men and FFMI <15 kg/m^2 for women).

Quadriceps endurance and strength. Isokinetic muscle endurance and strength of the dominant knee extensor (quadriceps muscle) were measured using a dynamometer (Biodex System; Biodex, Shirley, NY). Subjects were seated upright on the chair of the dynamometer with the back supported. Subjects were secured with straps at the level of the chest, pelvis, and thigh. The hip joint was at an angle between 90 and 100° of flexion during testing. The test consisted of 30 sequential volitional maximal contractions at an angular velocity of 90°/s, during which the subject was strongly encouraged. Maximal isokinetic strength was defined as the highest peak torque (in N·m) in this series of 30. To determine isokinetic quadriceps endurance, the proportional decline in peak torques (relative to the highest peak torque) per repetition (%N·m/rep) was used as determined by linear regression, as described previously (11). We used this slope as a measure of quadriceps endurance (i.e., a slope closer to zero indicates a better endurance). Valid quadriceps endurance measures were available for 24 patients with COPD and 13 healthy controls.

Daily physical activity. Physical activity (PA) was measured using a dual-axis GT1M accelerometer (ActiGraph; Fort Walton Beach, FL) for 6 consecutive days (4 weekdays and 2 weekend days). Subjects were instructed to wear the accelerometer during the time they were not asleep, except when showering or bathing (46). The accelerometer was firmly attached to an elastic belt worn at the waist. The accelerometer registers PA in counts, which are the summation of the accelerations measured during a specified time interval (epoch), which was set at 1 min. Counts represent the intensity of activity in that epoch. Non-weartime was defined as 60 consecutive counts of 0 in which up to two epochs <100 counts were allowed (41). Only days with ≥10 h of weartime were accepted as valid days (41). The total amount of PA was expressed as the total counts divided by the total weartime (counts/min). Cutoff points for sedentary lifestyle and combined moderate-to-vigorous PA (MVPA) intensity levels were defined as <100, $100–759$, and ≥760 counts/min, respectively (20, 41). The time spent in each category of intensity was presented as a percentage of total weartime. Furthermore, we analyzed the number, duration, and intensity of MVPA bouts (≥10 consecutive min spent in MVPA) (19). The classification of MVPA bouts was motivated by the PA recommendations of the Centers for Disease Control and Prevention, the American College of Sports Medicine (26), and the British Association of Sport and Exercise Sciences (24), which call for MVPA to be accumulated in bouts of ≥10 min to achieve health benefits. Moreover, these recommendations call for at least 150 min/wk of bouted MVPA. We investigated whether the participants in our study complied with these recommendations, accounting for the number of valid accelerometry days.

Skeletal muscle biopsy. Biopsies of the quadriceps muscle (vastus lateralis) from the dominant leg were obtained using a needle biopsy technique (8). Muscle tissue was frozen in melting isopentane precooled in liquid nitrogen and stored at $-80°$C for histological purposes. Another specimen was snap-frozen in liquid nitrogen and stored at $-80°$C for gene and protein expression analyses and for enzyme activity assays.

Fiber size and composition analysis. Serial cryosections (5 μm) with fibers in transverse orientation were cut from OCT-embedded muscle biopsies on a cryostat microtome (Leica CM 1900; Meyer Instruments) at $-20°$C and mounted on SuperFrost microscope slides (Menzel-Glüser, Braunschweig, Germany) to be stored at $-80°$C until further analyses. For immunohistochemistry, sections were incubated with primary antimyosin heavy chain (MyHC)-I [Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Ames, IA], anti-MyHC-IIa (DSHB), and anti-laminin (Sigma, Zwijndrecht, The Netherlands) followed by secondary antibodies labeled with Alexa Fluor 555, Alexa Fluor 488, and Alexa Fluor 350 (Invitrogen, Breda, The Netherlands). Fiber typing was aided by means of myosin ATPase-activity staining with acidic preincubation at pH 4.40 (25). Immunofluorescence-stained and ATPase-stained sections were microscopically photographed at 10× magnification. In a blinded fashion, fibers were classified primarily on the basis of immunofluorescence, with ATPase-stained sections used to confirm type I/IIa and type IIa/IIx hybrid fiber types. Fiber cross-sectional area was measured with Lucia 4.82 software (Laboratory Imaging, Prague, Czech Republic) on the basis of laminin staining of the basement membrane (45).

RNA extraction and RT-qPCR analysis. Muscle tissue (10–30 mg) was homogenized in denaturation solution (ToTALLY RNA Kit; Ambion, Foster City, CA) using a Polytron PT 1600 E (Kinematica, Littau/Luzern, Germany) and was RNA-extracted according to the supplier's protocol followed by genomic DNA removal and cleanup with the RNeasy Mini Kit with RNase-free DNase (Qiagen, Venlo, The Netherlands). After elution, RNA concentration was determined using a spectrophotometer (NanoDrop ND-1000; Isogen Lifescience, Ijsselstein, The Netherlands) and integrity was verified for a selection

of samples by gel electrophoresis and on a bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). RNA (400 ng) was reverse transcribed to cDNA with anchored oligo(dT) primers according to the supplier's protocol (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics, Woerden, The Netherlands). RT-qPCR primers were designed on the basis of Ensembl transcript sequences or selected from literature (3, 44) and ordered from Sigma Genosys (Zwijndrecht, The Netherlands). The genes encoding PGC-1α, PGC-1β, PGC-related coactivator (PRC), PPAR-α, Tfam, Nuclear Respiratory Factor (NRF)-1, NRF-2α, MyHC-I, muscle phosphofructokinase (PFKM), MyHC-IIa, and MyHC-IIx were the target genes. RT-qPCR reactions contained SensiMix SYBR Hi-ROX Kit (Quantace-Bioline, London, UK) with 300 nM primers and were run in a 384-well MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Standard curves prepared from pooled cDNA and melt curves were analyzed to verify efficiency and specificity of amplification. Twelve reference genes (ALAS1, ACTB, B2M, PPIA, GAPDH, GUSB, HMBS, HPRT1, RPL13A, RPLP0, UBC, YWHAZ) were measured and stability of expression was assessed by visual inspection of expression differences between the study groups and a stability assessment by geNorm (44). Eventually, nine reference genes (all except ACTB, GAPDH, and UBC) were used to calculate a geNorm factor, which was used to normalize expression levels of the target genes.

Immunoblotting of OXPHOS subunits and oxidative stress markers. Tissue (10–20 mg) was crushed in liquid nitrogen and homogenized in 400 μl of IP lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P40, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 5 mM NaF, 10 mM β-glycerophosphate, 1 mM Na$_4$O$_7$P$_2$, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1% aprotinin, 1 mM PMSF, pH 7.4) with a Polytron PT 1600 E (Kinematica). After homogenization, samples were incubated for 15 min on a rotating wheel at 4°C and spun for 30 min at maximum speed (20,817 $\times$ g) in a centrifuge cooled to 4°C. Supernatant was aliquoted, snap-frozen, and stored without sample buffer at −80°C until analysis.

Protein concentration in lysates was determined using the bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Breda, The Netherlands). For Western blot analysis, aliquots were supplemented with 4$\times$ sample buffer [250 mM Tris-HCl pH 6.8, 8% (wt/vol) sodium dodecyl sulfate, 40% (vol/vol) glycerol, 0.4 M dithiothreitol, 0.02% (wt/vol) bromophenol blue] and kept on ice. Per sample, 5 μg unboiled protein was separated on gel (4–12% Bis-Tris XT gel; Criterion, Bio-Rad Laboratories, Hercules, CA) with XT MOPS running buffer (Bio-Rad). One sample was loaded on all gels to facilitate gel-gel comparisons. Proteins were transferred to a 0.45-μm nitrocellulose membrane (Protran; Schleicher and Schuell, 's-Hertogenbosch, The Netherlands) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol] by electrophoresis. After transfer, membranes were blocked from nonspecific protein binding with blocking solution, which contains 5% (wt/vol) nonfat dry milk (Campina, Eindhoven, The Netherlands) in Tris-buffered saline with Tween20 [TBST; 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.05% (vol/vol) Tween20, pH 7.4], for 1 h at room temperature, followed by incubation in primary antibody solution overnight at 4°C [mouse anti-Total OXPHOS Rodent WB Antibody Cocktail (MS604–300; Abcam, Cambridge, UK) diluted 1:1,000 in blocking solution or rabbit anti-GAPDH (#2118; Cell Signaling Technology, Leiden, The Netherlands) diluted 1:5,000 in TBST]. Membranes were incubated in secondary antibody solution [peroxidase-labeled horse anti-mouse IgG or goat anti-rabbit IgG (PI-2000 and PI-1000, respectively; Vector Laboratories, Burlingame, CA] diluted 1:5,000 in blocking solution) for 1 h at room temperature before incubation with enhanced chemiluminescence substrate (Pierce SuperSignal West PICO Chemiluminescent Substrate; Thermo Fisher Scientific). Protein bands were detected using Super RX films (Fujifilm, Düsseldorf, Germany) and scanned on a GS-800 densitometer (Bio-Rad). Bands were quantified

using Quantity One software (v4.6.2; Bio-Rad), with GAPDH as loading control (GAPDH levels were not different between patients with COPD and controls).

Protein content of oxidative stress markers was identified using specific primary antibodies for protein carbonylation (anti-2,4-DNP moiety antibody, Oxyblot kit; Chemicon International, Temecula, CA), total protein nitration (anti-3-nitrotyrosine antibody, Invitrogen, Eugene, OR), and total malondialdehyde (MDA)-protein adducts (anti-MDA antibody; Academy Biomedical, Houston, TX). For protein carbonylation, carbonyl groups in the protein side chains were first derivatized to 2,4-dinitrophenylhydrazone (DNP) using the Oxyblot kit (Chemicon) according to the manufacturer's instructions. Briefly, 15 μg of protein was used per derivatization reaction; proteins were then denatured by addition of 12% SDS. The samples were subsequently derivatized by adding 10 μl of 1$\times$ 2, 4-dinitrophenyl-hydrazine solution and incubated for 20 min. Finally, 7.5 μl of neutralization solution and 2-mercaptoethanol were added to the sample mixture. Immunoblotting was similar as described above with some minor differences: polyvinylidene difluoride (PVDF) membranes were used, which were scanned with the Molecular Imager Chemidoc XRS System (Bio-Rad) and bands were quantified using the software Image Lab version 2.0.1 (Bio-Rad). Values of total reactive carbonyl groups, total protein tyrosine nitration, and total MDA-protein adducts in a given sample were calculated by addition of optical densities (arbitrary units) of individual protein bands in each case. Final optical densities obtained in each specific group of subjects corresponded to the mean values of the different samples (lanes) of each antigen studied. Actin (anti-alpha-sarcomeric actin antibody, clone 5C5; Sigma-Aldrich, St. Louis, MO) was used as the loading control for all the oxidative stress markers (10, 34).

Enzyme activity assays. Tissue (15–30 mg) was crushed in liquid nitrogen and homogenized in 350 μl SET buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4) with a Polytron PT 1600 E (Kinematica). After homogenization, samples were incubated for 10 min on a rotating wheel at 4°C and spun for 5 min at maximum speed (20,817 $\times$ g) in a centrifuge cooled to 4°C. An aliquot of supernatant was stored for protein determination. To the remaining supernatant, 5$\times$ aqueous BSA solution was added to a final BSA concentration of 1% (vol/vol) and samples were aliquoted, snap-frozen, and stored at −80°C until analysis. Protein concentration in SET lysate aliquots without BSA was determined using the bicinchoninic acid assay (Pierce). Citrate synthase (CS, EC 2.3.3.1), 3-hydroxyacyl-CoA dehydrogenase (HADH, EC 1.1.1.35) and PFK (EC 2.7.1.11) activities were assayed spectrophotometrically (Multiskan Spectrum; Thermo Labsystems, Breda, The Netherlands) as previously described (7, 18, 39). Absolute CS, HADH, and PFK activities were normalized to total protein.

Statistics. Differences between patients with COPD and controls were tested using Student's *t*-tests, Mann-Whitney-*U* tests, or χ^2 tests as appropriate. Correlations were tested using the Pearson correlation coefficient or the Spearman ρ in case of non-normally distributed data. Analyses were performed using PASW Statistics 17.0 (SPSS, Chicago, IL). A *P* value < 0.05 was considered statistically significant.

RESULTS

The control subjects were matched to the patients with COPD on the basis of age, sex, and BMI (Table 1). FEV$_1$ was between 30 and 50% of the predicted value in 13 patients (45%), between 50–70% of predicted value in 11 patients (38%), and >70% of predicted value in 5 patients (17%). $\dot{V}$o$_2$ peak and W peak were significantly decreased in the patients with COPD (Table 1). FEV$_1$ (% of predicted) was strongly correlated with $\dot{V}$o$_2$ peak (% of predicted) in the total population (r = 0.83, P < 0.001), within the group of patients with

Table 1. *Main characteristics of healthy controls and patients with COPD*

	Controls ($n = 15$)	Patients ($n = 29$)
Demographics		
Age, yr	65 (6)	65 (6)
Sex, % men	60	55
Ever-smoker, %	53	100†
BMI, kg/m^2	24.9 (3.3)	25.5 (3.6)
Pulmonary function		
FEV$_1$, %pred	113 (15)	58 (16)†
FVC, %pred	120 (17)	104 (22)*
FEV$_1$/FVC, %	74 (5)	45 (11)
DLCO, %pred	95 (19)	53 (18)†
Incremental load test		
Peak VO$_2$, %pred	123 (8)	72 (4)†
Peak WR, %pred	133 (7)	61 (4)†
ΔBorg dyspnea score	3.4 (0.8)	3.6 (0.4)
ΔBorg leg fatigue score	2.7 (0.6)	2.3 (0.4)
ΔPaO$_2$, kPa	1.04 (0.53)	−0.38 (0.31)*
ΔLactate/WR (mmol·1·W)	0.039 (0.003)	0.035 (0.003)

BMI, body mass index; DLCO, diffusion capacity of the lungs for carbon monoxide; FEV$_1$, forced expiratory volume in 1 s; FVC, forced vital capacity; WR, work rate. *$P < 0.05$, †$P < 0.001$.

COPD ($r = 0.78$, $P < 0.001$), and within the controls ($r = 0.53$, $P = 0.044$).

Quadriceps muscle endurance and markers of oxidative phenotype. Quadriceps muscle endurance was significantly lower in the patients with COPD (Fig. 1*A*). The mean decline in peak torque per repetition was $-1.67 \pm 0.09\%$ N·m/rep in patients with COPD vs. $-1.18 \pm 0.10\%$ N·m/rep in the controls ($P = 0.002$). Patients had a lower proportion of type I fibers and a higher proportion of type IIa, type IIa/IIx, and IIx fibers than controls, indicative of a I-to-II fiber type shift (Fig. 1, *B–D*). The mean mRNA expression levels of the key regulator of oxidative metabolism PGC-1α and of the structural marker of oxidative fibers, MyHC-I, were significantly decreased in the patients with COPD compared with controls, and reached borderline significance for Tfam (Fig. 1*E*). Other (co-)transcription factors implicated in driving oxidative gene expression (PPAR-α, PGC-1β, PRC, NRF-1, and NRF-2α) were not differentially expressed between the patient and control groups. Gene expression levels of the key glycolytic enzyme PFKM and of the structural markers of glycolytic fibers (i.e., MyHC-IIa and MyHC-IIx) were significantly higher in patients with COPD (Fig. 1*F*). Congruently, mean OXPHOS subunit protein levels were all lower in patients with COPD than in controls, reaching statistical significance for those of complexes IV and V (Fig. 1, *F* and *G*). The mean differences in HADH, CS, and PFK enzyme activity were not

statistically significant (Fig. 1*H*), yet the ratio HADH/PFK was significantly lower in patients with COPD. The ratio CS/PFK reached borderline significance (Fig. 1*I*).

Quadriceps strength, muscle mass, and fiber cross-sectional area. Neither FFMI nor quadriceps peak torque were significantly different between the patients with COPD and controls (Fig. 2, *A* and *B*). The prevalence of muscle wasting was not different between patients with COPD and controls (20.7% vs. 13.3%, respectively; $P = 0.70$). Congruently, there were no differences in muscle fiber CSA (Fig. 2*C*).

Daily living physical activity level. From all the subjects, 5.5 ± 0.9 valid accelerometry days were available with a mean of 14 ± 1 h of wear time per day. Total PA was significantly lower in patients with COPD than controls, and patients spent more time in sedentary behavior and less time in MVPA (Table 2). Also, patients had fewer MVPA bouts, which were shorter and less intense than the MVPA bouts of controls (Table 2). Total PA (counts/min) was positively correlated with FEV$_1$ (% of predicted) in the entire study population ($r = 0.58$, $P < 0.001$) and also within the group of patients with COPD ($r = 0.55$, $P = 0.002$) but not in the controls ($r = -0.06$, $P = 0.82$). Within the group of patients with COPD, FEV$_1$ (% of predicted) was negatively correlated with time spent in sedentary PA ($r = -0.54$, $P = 0.002$) and positively correlated with time spent in MVPA ($r = 0.61$, $P < 0.001$), number of MVPA bouts per day ($r = 0.46$, $P = 0.013$), and with time spent in MVPA bouts ($r = 0.41$, $P = 0.039$). Only 31% of the patients fulfilled the criteria of 150 min/wk of bouted MVPA time vs. 80% of the controls ($P = 0.004$). Within the patient group, those who fulfilled these criteria had a significantly higher FEV$_1$ compared with patients who did not fulfill these criteria ($67 \pm 5\%$ vs. $54 \pm 3\%$ of predicted; $P = 0.049$).

Associations with quadriceps oxphen in patients with COPD. In the group of patients with COPD, quadriceps endurance was significantly, positively correlated with MyHC-I gene expression and PGC-1α gene expression, and showed a trend toward a positive correlation with type I fiber proportion (Fig. 3, *A–C*). In line with this finding, type IIx fiber proportion was negatively correlated with quadriceps endurance ($\rho = -0.434$, $P = 0.038$). Moreover, type I fiber proportion was negatively correlated with the increase in lactate relative to work rate (Fig. 3*D*).

No significant correlations were found between markers of oxphen and quadriceps peak torque, pulmonary function, exercise capacity, total PA, time spent in sedentary PA or MVPA, or any of the MVPA bout-related variables (data not shown).

Skeletal muscle oxidative stress. The levels of protein carbonylation, tyrosine nitration, and MDA-protein adducts were not different between patients and controls (Fig. 4). Among the

Fig. 1. Quadriceps muscle endurance, fiber type distribution, gene expression, OXPHOS protein expression, and enzyme activity. *A*: quadriceps endurance as assessed by isokinetic dynamometry. The *P* value denotes the statistical significance of the difference in slopes between patients with COPD and healthy controls. *B*: quadriceps muscle fiber type distribution as determined by fluorescent immunohistochemistry and ATPase staining. *C*: box plot of fiber type IIa/IIx proportion. Boxes represent 25th and 75th percentiles; whiskers represent 10th and 90th percentiles. *D*: box plot of fiber type IIx proportion. Boxes represent 25th and 75th percentiles; whiskers represent 10th and 90th percentiles. *E*: quadriceps muscle mRNA expression of peroxisome proliferator-activated receptor (PPAR)-γ; coactivator-1α (PGC-1α); myosin heavy chain (MyHC) I, IIa, and IIx; mitochondrial transcription factor (Tfam); PPAR-α; PGC-1β; nuclear respiratory factor (NRF)-1; NRF-2α; PGC-related coactivator (PRC); and phosphofructokinase muscle (PFKM) as determined by RT-qPCR. *F*: representative Western Blot of the five OXPHOS protein subunits and GAPDH from three healthy controls and three patients with COPD. All bands are from the same blot and were reorganized for illustrative purposes because patients and controls were randomized over the blot and to respect the ascending order of the five subunits. *G*: levels of the five OXPHOS protein subunits as determined by Western Blot. *H*: 3-hydroxyacyl-CoA dehydrogenase (HADH), citrate synthase (CS), and phosphofructokinase (PFK) enzyme activities. *I*: ratios between HADH/PFK and CS/PFK. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
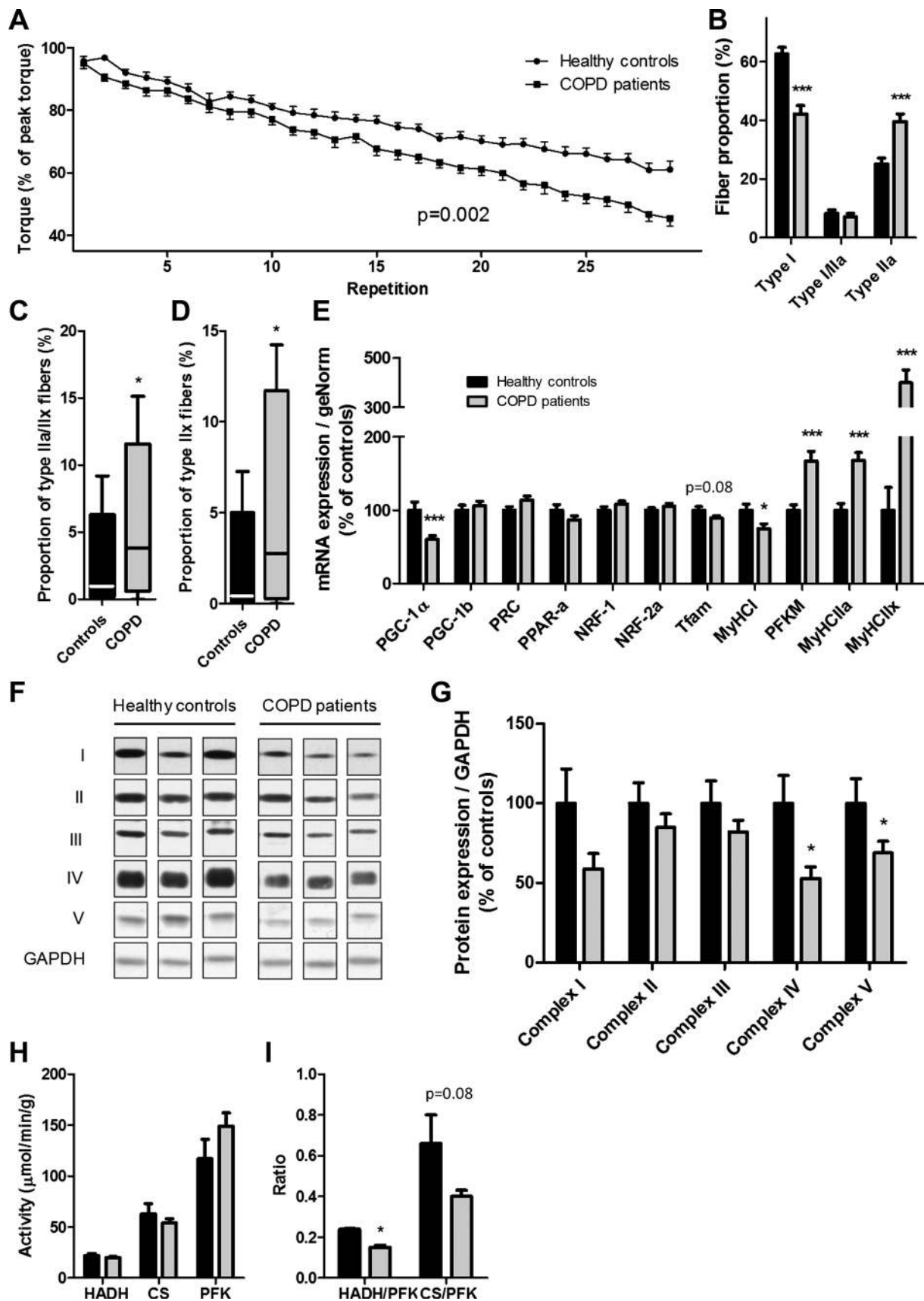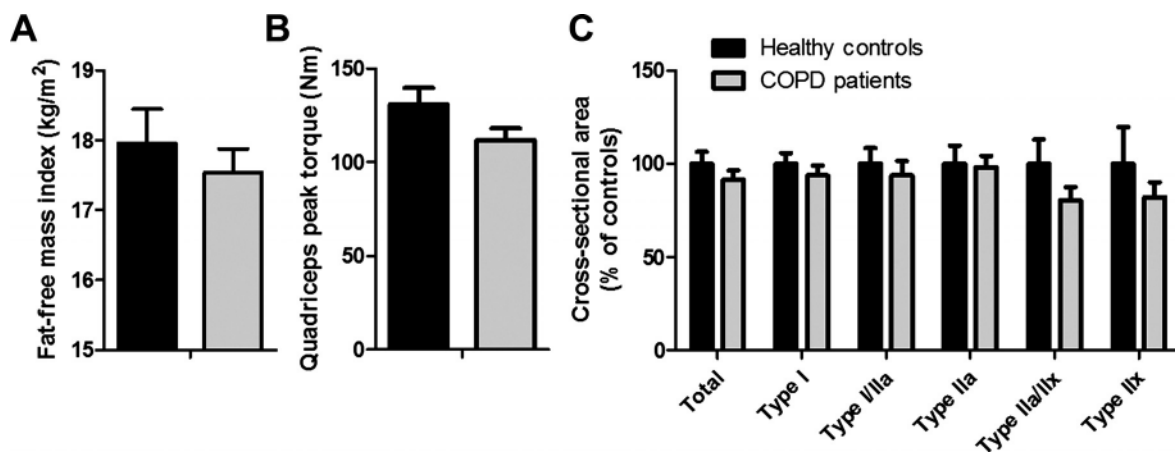
Fig. 2. Fat-free mass index, quadriceps strength, and fiber cross-sectional area. *A*: fat-free mass index as determined by whole-body dual-energy X-ray absorptiometry; $P > 0.05$. *B*: quadriceps peak torque as determined by isokinetic dynamometry; $P > 0.05$. *C*: quadriceps muscle fiber cross-sectional areas presented for total fibers and per fiber type; all $P > 0.05$.

patients with COPD, we found inverse correlations between protein carbonylation and type I fiber proportion, MyHC-I gene expression, and quadriceps endurance, and a positive correlation between protein carbonylation level and MyHC-IIa expression (Fig. 5). In line with this finding, protein carbonylation was positively correlated with type IIx fiber proportion ($\rho = 0.429$, $P = 0.023$). Additionally, tyrosine nitration was inversely correlated with quadriceps endurance ($r = -0.422$, $P = 0.040$), and MDA-protein adducts were inversely associated with MyHC-I gene expression ($r = -0.384$, $P = 0.044$). Markers of oxidative stress were not correlated with FFMI or fiber CSA among patients with COPD, and no correlations were found between oxidative stress markers and markers of oxphen in the healthy subjects (data not shown).

DISCUSSION

Whereas previous studies have clearly shown skeletal muscle dysfunction in patients with advanced COPD, the main novelty of the current study is that we identified a loss of quadriceps oxphen and decreased quadriceps endurance in patients with mild-to-moderate COPD. Interestingly, these abnormalities existed even in the absence of significant muscle wasting. Also, we found consistent, inverse correlations between markers of oxphen and oxidative stress among the patients with COPD. Note that our study was of cross-sectional nature and did not allow for longitudinal inferences. However,

linking the various cross-sectional studies across COPD severity stages, the available data suggest that the loss of skeletal muscle endurance and skeletal muscle oxphen already occur in patients with mild-to-moderate COPD and progress with declining lung function, and suggest a potential involvement of oxidative stress.

We assessed a large panel of markers of skeletal muscle oxphen and found that many were significantly decreased in our patients with mild-to-moderate COPD compared with well-matched controls. More specifically, the loss of oxphen in our group of patients with COPD was characterized by a decreased proportion of type I fibers, decreased gene expression of PGC-1α and MyHC-I, decreased protein expression of subunits of OXPHOS complexes IV and V, and decreased HADH/PFK enzyme activity. The mean differences in proportions of type I, IIa, and IIx fibers between our patients with mild-to-moderate COPD and matched healthy controls were 20%, 15%, and 4%, respectively. For comparison, pooled analyses from a meta-analysis in patients with advanced COPD showed these differences to be 22%, 7%, and 13%, respectively (15). These combined data suggest that a decrease in slow-oxidative type I fiber proportion already occurs in the early stages of COPD, and that a further shift from fast-oxidative type IIa to fast-glycolytic type IIx fibers continues in advanced COPD toward even more dependence on glycolytic metabolism. It should be acknowledged that decreased expres-

Table 2. *Accelerometry data from patients with COPD and controls*

	Controls ($n = 15$)	Patients ($n = 29$)
Total activity, counts/min	360 (134)	220 (101)‡
Time spent in sedentary PA, % of total wear time	60.5 (6.6)	67.4 (9.4)†
Time spent in lifestyle PA, % of total wear time	25.5 (4.6)	24.3 (6.5)
Time spent in MVPA, % of total wear time	13.8 (5.3)	8.6 (5.3)†
Total number of MVPA bouts	12 (8–18)	5 (3–11)†
Number of MVPA bouts per day	2.0 (1.3–2.0)	1.0 (0.6–2.2)*
Time spent in MVPA bouts, min	267 (108–494)	68 (38–204)†
Mean MVPA bout duration, min	22.1 (9.4)	14.7 (3.7)†
Mean intensity of MVPA bouts, counts[a]	2700 (605)	1913 (498)‡
Compliance with MVPA recommendation, %[b]	80	31†

MVPA, moderate-to-vigorous physical activity; PA, physical activity. [a]Weighted for individual MVPA bout duration. [b]At least 150 min/wk of bouted MVPA time. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.
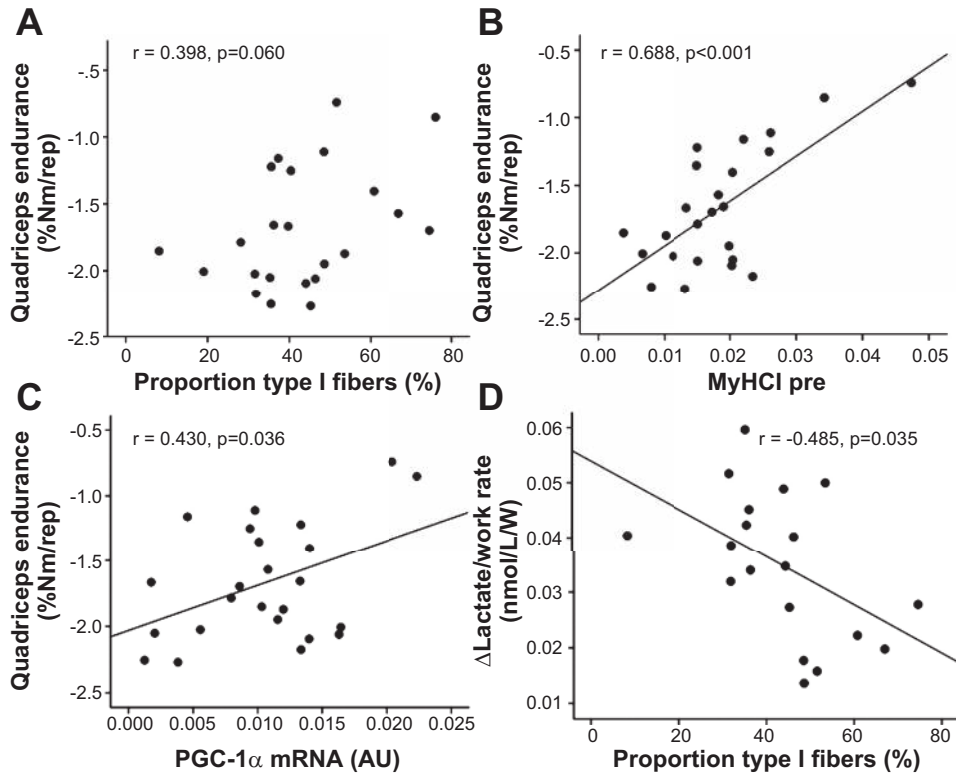
Fig. 3. Correlations among patients with COPD between markers of skeletal muscle oxidative phenotype and quadriceps endurance, and the rise in blood lactate relative to work rate during an incremental cycling test. *A*: correlation between the proportion of type I fibers and quadriceps endurance. *B*: correlation between myosin heavy chain I mRNA expression and quadriceps endurance. *C*: correlation between PGC-1α mRNA expression and quadriceps endurance. *D*: correlation between the proportion of type I fibers and the rise in blood lactate relative to work rate during an incremental cycling test.

sion of subunits of the key respiratory chain complexes does not necessarily indicate decreased cytochrome c oxidase (COX) activity or overall lower mitochondrial respiration. Several other studies have assessed COX activity in muscle biopsies of patients with COPD and showed contrasting results. For example, reduced skeletal muscle COX activity has been reported in patients with moderate COPD by some authors (14, 16, 23), whereas others reported even higher COX activity in patients with moderate-to-severe COPD (30, 36). Between-study differences remain unexplained; therefore, these data provide further indication for studying mitochondrial respiration in more detail in different COPD disease stages, including the early stages.
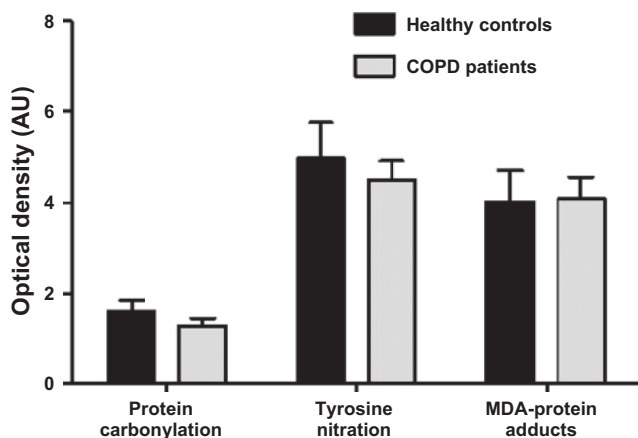

Fig. 4. Levels of skeletal muscle oxidative stress. The levels of protein carbonylation, tyrosine nitration, and MDA-protein adducts were not different between patients with COPD and controls; all $P < 0.05$.

We found that markers of oxidative phenotype were significantly correlated with quadriceps endurance, implying that the magnitude of loss of oxidative phenotype reached the level of clinical importance in terms of quadriceps endurance. Similar findings were previously reported in a population of patients with advanced COPD with significant muscle wasting (2). Our data show that these abnormalities are already present in early COPD even in the absence of muscle wasting. In a recent study by Saey et al. (35), the change in lactate/work rate during a constant work rate test was significantly higher in patients with COPD and muscle wasting (mean FEV_1 45% of predicted) compared with controls. This coincided with a decreased proportion of type I fibers and increased type IIa fibers, and a wide array of increased glycolytic markers. The authors argued that increased glycolysis underlies the increased change in lactate/work rate. Although this seems plausible, no correlations between the change in lactate/work rate and metabolic markers were presented by Saey et al. (35). We did, however, find that the proportion of type I fibers was associated with an increased change in lactate/work rate during an incremental load test in patients with COPD without muscle wasting.

PGC-1α is considered the major regulator of skeletal muscle oxidative metabolism. Indeed, its expression is known to be significantly induced upon aerobic exercise, which subsequently orchestrates oxidative gene expression to prepare the muscle for a next bout of exercise (28). Puente-Maestu et al. (29) have recently shown that moderate-intensity exercise increased skeletal muscle PGC-1α gene expression in patients with COPD (FEV_1 50% of predicted) with normal FFMI. Because we found that PGC-1α gene expression was decreased by 40% in our group of patients, this would suggest that the muscle oxidative machinery fails, at least in part, at the regulatory level. The downstream target of

Fig. 5. Correlations among patients with COPD between markers of skeletal muscle oxidative phenotype, quadriceps endurance, and level of protein carbonylation. Correlations between the level of protein carbonylation and proportion type I fibers (*A*), quadriceps endurance (*B*), myosin heavy chain mRNA expression (*C*), and myosin heavy chain IIa mRNA expression (*D*).
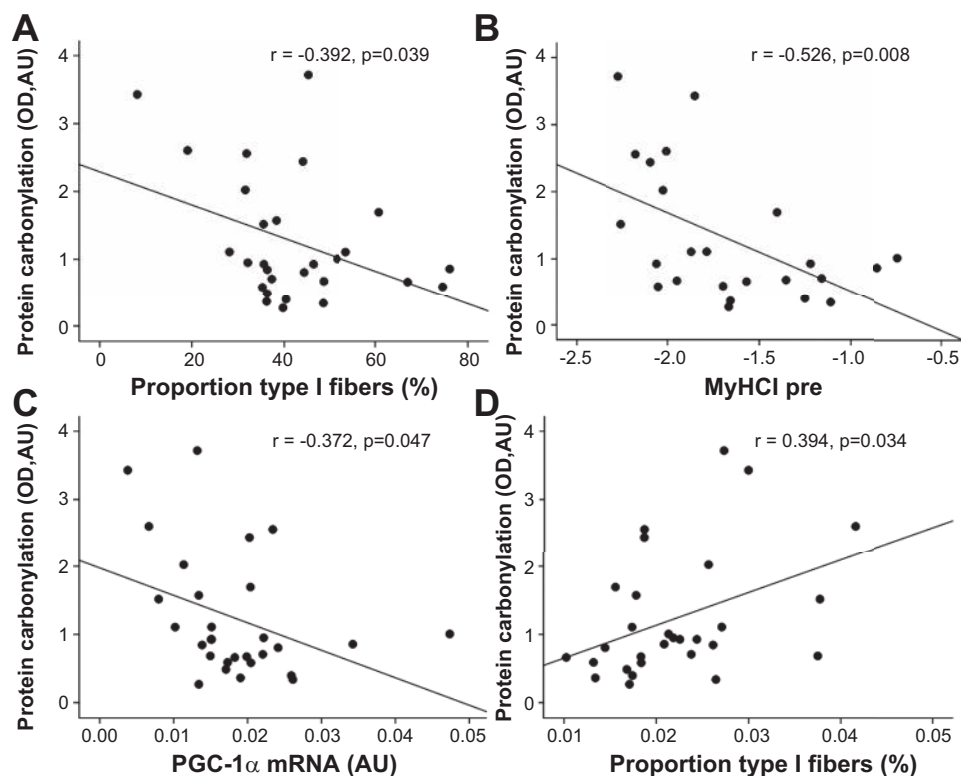
PGC-1α, Tfam, tended to be decreased expressed as well. Expression levels of other co-transcription factors implicated in the regulation of Tfam expression (e.g., PGC-1β, PRC, NRF-1, and NRF-2α) were not differentially expressed between patients with COPD and controls.

Physical inactivity, or deconditioning, has been proposed to contribute to skeletal muscle dysfunction in COPD (47). We used accelerometry to assess both quantitative and qualitative daily living physical activity, and studied the relations with muscle oxphen. We did not specifically select the healthy subjects on the basis of sedentary behavior, so the finding that our patients with COPD were on average 39% less physically active compared with the controls was not surprising. In addition, we found that our group of patients engaged in less MVPA bouts and that these bouts were shorter and less intense than those of controls. The level and intensity of physical activity was positively correlated with FEV$_1$ within the group of patients with COPD, which is consistent with previous reports (13, 17, 40). Interestingly, however, we could not identify any relation between markers of oxphen and total physical activity level nor its intensity in our group of patients. Although we did not select our healthy controls on the basis of sedentary behavior, our data do not support the presumption that physical inactivity is the principal determinant of impaired skeletal muscle oxphen in mild-to-moderate COPD. Still, the fact that no association between loss of oxphen and physical inactivity was found does not exclude the possibility that physical activity plays a role in the multifactorial pathways involved in loss of oxphen.

Whereas basal levels of skeletal muscle oxidative stress were comparable between our patients and controls, we found that markers of muscle oxidative stress were significantly associated with the loss in muscle oxphen among patients with COPD, but were not related to muscle mass. Oxidative stress has been suggested to render the muscle susceptible to atrophy because damaged (oxidized) proteins are prone to be degraded. However, this concept has recently been challenged because no differences in basal skeletal muscle oxidative stress levels were found between patients with advanced COPD with and without muscle wasting (10). Enhanced mitochondrial production of reactive oxygen species (ROS) has been observed in patients with moderate-to-severe COPD, which has been proposed to be linked to the proportional increase in type II fibers (27). Recently, it has also been demonstrated that OXPHOS complex III is the main site of ROS production within peripheral muscles of patients with COPD, who exhibited similar clinical features as those recruited in the current investigation (31). Furthermore, compared with type I fiber mitochondria, type II fiber mitochondria exhibit enhanced ROS production, as was shown in rats (4). Taken together, the associations encountered between protein oxidation levels and the slow-to-fast phenotype shift among patients with mild-to-moderate COPD may be partly explained by mitochondrial dysfunction and enhanced ROS production.

In peripheral skeletal muscle of emphysematous hamsters, decreased citrate synthase activity and increased lipid peroxidation have been reported, which were not related to decreased body weight, muscle mass, or physical activity level (21, 22). In line with these findings, in a rat model of emphysema, Zhang et al. (48) reported no change in muscle mass but showed increased skeletal muscle lipofuscin inclusions (a marker of oxidative stress), which was correlated with decreased muscle endurance. Collectively, the available data suggest a relation between oxidative stress and the loss of oxidative capacity in COPD-related muscle dysfunction and it can be speculated that loss of oxphen, through enhanced oxidative stress, may eventually augment muscle wasting as well.

In conclusion, this study shows evidence for a loss of muscle oxphen and decreased quadriceps endurance in patients with mild-to-moderate COPD without overt loss of muscle mass. Our results indicate that timely intervention strategies aimed at improving muscle oxphen in early COPD may improve or prevent a further loss in quadriceps endurance.

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